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(54) Title: DNA ENCODING A HUMAN CALCIUM CHANNEL ALPHA-1E SUBUNIT

(57) Abstract

Isolated DNA encoding a human neuronal calcium channel alpha-1 subunit of subtype E and its corresponding polypeptide are disclosed. Also disclosed are cells expressing the human neuronal calcium channel alpha-1E subunit, as well as methods for screening for therapeutic compounds, using such compositions.

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DNA ENCODING A HUMAN CALCIUM CHANNEL ALPHA-1E SUBUNIT

Field of the Invention

The present invention relates to human calcium channel compositions. In particular, the invention includes compositions containing a human neuronal calcium channel alpha subunit designated subtype "1E" herein. Compositions of the invention include coding sequences for the subunit, as well as cells containing such coding sequences and expressing the human neuronal alpha-1E subunit.

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35

Background of the Invention

Voltage-gated calcium channels are present in a wide variety of tissues, particularly excitable tissues, where they act to regulate membrane

excitability. Such functions as muscle contraction and synaptic transmission are regulated, at least in part, by specific voltage-gated calcium channels. Compounds, such as 5 dihydropyridine compounds, that block certain of these channels are used therapeutically in the management of various disorders such as hypertension, angina and subarachnoid hemorrhage.

Voltage-gated calcium channels have generally 10 been classified according to their electrophysiological and pharmacological properties. Currently, at least four classes of voltage-gated calcium channels are recognized: L-type, T-type, N-type, and P-type. However, 15 molecular cloning techniques have revealed the existence of different sub-types of channel within some of these classes. (see Tsien et al., Trends in Pharmacological Sciences 12: 349-354, for review).

20 In general, voltage gated calcium channels have been shown to consist of at least 4 non-identical subunits: the alpha-1 subunit, alpha-2 subunit, beta subunit and gamma subunit. For the L-type calcium channels, which are probably the 25 best characterized calcium channels, it has been shown that the alpha-1 subunit contains the binding site for dihydropyridine ligands.

30 Partial cDNA clones encoding portions of several different subtypes of the rat neuronal calcium channel alpha-1 subunit, referred to as rat brain class A, B, C and D, were first isolated from rat brain cDNA libraries (Snutch, et al., 1990). Homologous alpha-1 clones subtypes A-D have been described in humans by Harpold et al. 35 (PCT/US92/06903; WO93/04083).

The present invention is concerned with a fifth human neuronal calcium channel alpha-1

subunit, termed "1E" or α -1E herein. This subunit diverges considerably from the other human alpha-1 subunit clones, exhibiting only about 62% deduced amino acid sequence homology to other 5 human neuronal alpha-1 subunits. As shown herein, this subunit can form a heterologous calcium channel with alpha-2 and beta subunits. The novel channel has a unique pharmacology and is therefore 10 useful in screening for new, more selective therapeutic agents directed at calcium channel modulation.

Summary of the Invention

In one aspect, the invention includes an 15 isolated DNA fragment that encodes an alpha-1E subunit of a human neuronal calcium channel. The fragment has the nucleotide sequence presented as SEQ ID NO:1.

Also included in the invention is an alpha-1E 20 polypeptide subunit of a human neuronal calcium channel having the sequence presented as SEQ ID NO:2. In another aspect, the invention includes a mammalian expression vector containing the nucleotide sequence SEQ ID NO:1.

25 A eukaryotic cell in accordance with the invention includes a heterologous DNA having the sequence presented as SEQ ID NO:1. The cell expresses a neuronal calcium channel including a polypeptide subunit having the sequence SEQ ID NO: 30 2. The cells may be further designed to express neuronal calcium channel subunits α , β , and γ .

The cell line can be used to screen compounds effective to inhibit calcium uptake by neuronal cells.

35 These and other objects and features of the invention will be more fully appreciated when the following detailed description of the invention is

read in conjunction with the accompanying drawings.

Brief Description of the Drawings

5 Figure 1 shows the DNA sequence (SEQ ID NO: 1) of the human neuronal calcium channel alpha subunit α -1E and the deduced amino acid sequence (SEQ ID NO: 2) for the channel subunit; and

10 Figure 2 shows a tracing of a barium current measured in a eukaryotic cell expressing a heterologous calcium channel including the human neuronal calcium channel alpha subunit α -1E.

Detailed Description of the Invention

15 I. Definitions

Unless otherwise indicated, all terms used herein have the same meaning as they would to one skilled in the art of the present invention.

Practitioners are particularly directed to 20 Current Protocols in Molecular Biology (Ausubel) for definitions and terms of the art.

The terms "heterologous DNA" and "heterologous RNA" refer to nucleotides that are not endogenous to the cell or part of the genome 25 in which they are present; generally such nucleotides have been added to the cell, by transfection, microinjection, electroporation, or the like. Such nucleotides generally include at least one coding sequence, but this coding sequence need not be expressed.

The term "expression vector" refers to 30 vectors that have the ability to incorporate and express heterologous DNA fragments in a foreign cell. Many prokaryotic and eukaryotic expression vectors are commercially available. Selection of 35 appropriate expression vectors is within the knowledge of those having skill in the art.

II. Isolation of DNA Coding Sequences for Human Neuronal Alpha-1E Subunit

The diversity of voltage-gated calcium channels between and within tissues as well as across species is becoming more apparent, as new channels having distinct electrophysiological and pharmacological characteristics are reported. The present invention describes a new calcium channel isolated from human brain. As reported herein, this channel is categorized as an alpha subunit of a voltage-gated calcium channel. The novel human channel reported herein is referred to as the human alpha-1E subunit, or α -1E, in accordance with the nomenclature defined by Snutch for rat brain-derived calcium channel alpha subunits A-D.

A number of overlapping partial cDNA clones were isolated from a human hippocampal library in order to characterize the complete α -1E coding sequence. The complete α -1E nucleotide (nt) sequence is depicted in Figure 1 as SEQ ID NO: 1. Also shown is the deduced amino acid sequence (single letter code indicated below nt sequence) as SEQ ID NO: 2. A list of the of four partial cDNA clones used to characterize the α 1E sequence and the nucleotide position of each clone relative to the full-length α -1E sequence (SEQ ID No.1) is shown in Table 1 below.

Table 1

Identification of Partial α -1E Clones

Name	SEQ ID NO	Location Relative to Full-Length Sequence
H24	3	nt 1 to 2374 of SEQ ID No 1
H6	4	nt 1185 to 5377 of SEQ ID No 1
I2	5	nt 3192 to 6435 of SEQ ID No 1
3-69A	6	nt 5176 to 692 of SEQ ID No 1

The isolation and characterization of each of the partial clones are described below and detailed in Example 1. The codon for the start methionine begins with nt 107 and nt 6912 5 represents the end of the open reading frame. The initial 107 and final 10 nucleotides (nt) represent 5' and 3' untranslated regions, respectively.

10 A. Screening Libraries for Human Calcium Channels

Synthetic oligonucleotide probes for hybridization screening were prepared on an automated oligonucleotide synthesizer (Applied Biosystems, Foster City, CA). In order to obtain 15 clones to form the complete α -1E sequence, oligonucleotide probes based on other calcium channels were constructed as described in Example 1. Probes were constructed based on the sequence 20 of the rat calcium channels such as those described by Snutch and Dubel. These subunits are alpha-1 subunits of rat neuronal calcium channels. They exhibit only about a 62% amino acid sequence 25 identity with the α -1E clone which was eventually constructed. Further probes were restriction fragments of partial clone H6.

A human hippocampal library was obtained from a commercial supplier (Stratagene, La Jolla, CA). Alternatively, brain libraries from the 30 hippocampal or other discrete brain regions, or from the whole brain, can be produced according to standard methods now known in the art (Ausubel), in order to identify variants, such as splice variants of the α -1E channel. Such methods 35 include lysing of cells comprising the region of interest to extract RNA. PolyA+ RNA is then isolated and used to synthesize single-stranded cDNA, from which is produced double-stranded cDNA,

using specific or random hexamer primers, such as hexadeoxynucleotide primers (Clontech, Palo Alto, CA).

5 Restriction enzyme adaptor regions are then ligated to the single-stranded cDNA, according to standard methods (Sambrook). Such cDNA is then purified and size-selected by agarose gel chromatography. The cDNA is then eluted and ligated to a selected vector sequence, such as a 10 lambda gt11 vector or a lambda ZAP vector, as used in the Examples reported herein. The vector is then packaged into appropriate phage hosts and used to infect bacterial cells, as E.coli according to methods known in the art.

15 Commercial or custom generated libraries, such as phage-cDNA libraries described above, are screened using as probes oligonucleotide hybridization probes, as described in Example 1, under standard conditions, and medium stringency 20 (Ausubel). Briefly, oligonucleotide hybridization probes are radiolabeled by random priming methods (Sambrook), then hybridized to immobilized DNA from the cDNA library. Those phage plaques showing hybridization with the probes are 25 selected, subcloned, and re-tested. Positive clones are identified by autoradiography. Clones identified according to the methods described above are expected to be partial sequence clones, due to the size selection of the cDNA used in 30 generating the library and the predicted size of the calcium channel alpha subunit, based on data from calcium channels from other species.

35 Clones identified and isolated as described above are plaque purified prior to extraction of DNA and production of double stranded plasmid cDNA. The sequence of the plasmid cDNA is then determined, by standard methods, such as on a

commercial DNA sequencer (Applied Biosystems, Foster City, CA). As noted above, four partial clones, H6, H24, I2, and 3-69A, were used to determine the full-length sequence of α -1E.

5 Regions of overlap between different clones obtained were determined by comparison of the sequences.

Alternatively, the sequences provided by the end-terminal sequences of partial clones useful as 10 specific sequence primers in first-strand DNA synthesis reactions (Maniatis et al.; Scharf et al.) using, for example, partially purified total cellular RNA as substrate. Synthesis of the second-strand of the cDNA is randomly primed 15 (Boehringer Mannheim, Indianapolis IN). The above procedures identify or produce cDNA molecules corresponding to nucleic acid regions that are adjacent to any of the partial clone sequences. These newly isolated sequences can in 20 turn be used to identify further flanking sequences, and so on, to identify overlapping cDNA clones from which the entire α -1E sequence can be determined. Using the general methods described above, and detailed in Example 1, the sequence of 25 the full-length α -1E coding sequence is determined. The full-length α -1E clone is constructed from the partial overlapping clones is detailed in Example 2.

30 B. Construction of Full-length α -1E Clones

With reference to Table 1 and Figure 1, it can be seen that α 1E cDNA clones H24, H6, I2, and 35 3-69A, isolated as described above, overlap and include the nucleotide sequence which codes for the entire α 1E open reading frame, nt 107 to 6912 (SEQ ID No: 1). Restriction fragments of these partial cDNA clones were ligated together to

generate a full-length $\alpha 1E$ cDNA in a eukaryotic expression vector (pcDNA III- Invitrogen). The resulting construct was named NX-HE1.

The construction of $\alpha 1E$, termed NX-HE1 and identified as SEQ ID NO: 1, herein, was performed in multiple steps as described in detail in Example 2. Briefly, the H24 clone (SEQ ID NO:3) was recloned into Bluescript SK+ ("BS") (Stratagene, San Diego, CA) to obtain proper orientation within the BS polylinker such that the BS XhoI site was 5' relative to the coding region, forming Construct 1. An XhoI fragment from Construct 1 was then ligated into the eukaryotic expression vector pcDNA III (Invitrogen, San Diego, CA) to form Construct 2. An XhoI/ApaI fragment from clone H6 (SEQ ID NO: 4) was then ligated into Construct 2 form Construct 3. The remainder of the 3' end was constructed separately, as follows. An SfiI/BamHI fragment from clone 3-69A was ligated into clone I2 (SEQ ID NO: 5) to form Construct 4. Finally, an ApaI fragment of Construct 4 was ligated into Construct 3 to from the full length clone SEQ ID No.1). This ligated product containing the full length sequence was named NX-HE1.

III. Heterologous Expression of $\alpha 1E$ in Cells

It can be appreciated that, given the diversity and importance of voltage-gated calcium channels in mammalian physiology, possession of cells which transiently or constitutively express selected channel subtypes, such as the $\alpha 1E$ subtype calcium channel, would find use in the medical arts, particularly in the areas of diagnosis and/or drug screening. Exemplary assays in these areas are described in Section V.

It can be appreciated that functional expression of calcium currents is particularly useful in practicing parts of the invention described herein. However, expression of a 5 particular heterologous DNA sequence can be monitored to some advantage, using non-functional assays, such as Northern blot assays and protein expression assays, such as immunodetection methods. The present invention provides tools for 10 use in such methods, including oligonucleotide probes and proteins and peptides for production of antibodies for use in immunodetection assays.

15 A. Preparation of Recombinant Eukaryotic Cells Containing DNA Encoding Heterologous α -1E Subunits

DNA encoding the α -1E calcium channel subunit may be introduced into a host cell for expression of the DNA. Methods for introduction 20 of such DNA into cells are known those skilled in the art (Ausubel, Sambrook). Such methods include, for example, transfection of eukaryotic cells with an appropriate expression plasmid vector, such as the vectors described in Section 25 II herein, or a combination of plasmid vectors each containing a different calcium channel subunit, selected from alpha-1, alpha-2, beta, and gamma subunits known in the art to form functional calcium channels (Williams). each encoding one 30 or more distinct genes or with linear DNA, and selection of transfected cells are also well-known in the art (Sambrook, et al., 1989).

35 Cloned full-length DNA encoding the alpha-1E subunit of a human calcium channel, such as the α -1E DNA sequence SEQ ID NO: 1, described herein, introduced into a plasmid vector for expression in a eukaryotic cell. Host cells may also be

transfected with linear DNA according to standard methods.

Practice of the present invention can be effectively carried out using any of a number of mammalian expression systems, including yeast cells. However, mammalian expression systems may be preferred for practicing certain aspects of the invention.

Eukaryotic cells suitable for introduction of heterologous α -1E calcium channel subunit include any cells that are transfectable by such DNA or RNA or into which such DNA may be injected. Preferred host cells are those that can also express the heterologous DNA and RNA. For practicing certain aspects of the invention, such as electrophysiological measurements described in Section IV it is appreciated that it may be desirable that the host cell lack endogenous functionally expressed voltage gated calcium channels having current characteristics similar to those exhibited by the human alpha-1 calcium channel subunits described herein. In such cases it may be necessary, in order to observe and measure functional expression of the exogenously added alpha-1E subunit, to introduce into the cell heterologous coding sequences encoding the alpha-2 and possibly also the beta calcium channel subunit. As described above, coding sequences for such auxilliary subunits have been published, and expression in vectors suitable for introduction into cells is well within the skill of the practitioner.

In addition, preferred cells for introducing DNA include those that can be transiently or stably transfected and include, but are not limited to, cells of mammalian origin, such as COS cells, mouse 1 cells, Chinese Hamster Ovary (CHO)

cells, human embryonic kidney (HEK) cells, African green monkey cells, and the like. Preferred cells include DG44 cells and HEK 293 cells, particularly HEK 293 cells that have been adapted for growth in suspension. Additionally *Xenopus laevis* oocytes find use in the practice of the invention, as described in Part B and Section IV, below. Additionally, yeast cells such as *Saccharomyces cerevisiae* or *Pichia pastoris* may be utilized in practicing the invention.

Heterologous DNA encoding a human alpha-1E subunit, such as the α -1E calcium channel subunit, may be introduced by any method known to those skilled in the art, such as transfection with a vector containing the DNA sequence that encodes the subunit, such as the DNA sequence SEQ ID NO: 1. Particularly preferred vectors for transfection of mammalian cells are the pSV2dhfr expression vectors, which contain the SV40 early promoter, mouse dhfr gene, SV40 polyadenylation and splice sites and sequences necessary for maintaining the vector in bacteria, cytomegalovirus (CMV) promoter-based vectors such as pCMV or pCDNA1 or pCDNA3 (Invitrogen), and MMTV promoter-based vectors. DNA encoding the human calcium channel subunit α -1E is been inserted in the vector pCDNA3 such that its expression is regulated by the CMV promoter. Such constructs are suitable for transfecting a number of mammalian cells, including COS cells and HEK 293 cells. Other suitable vectors and cell targets include, but are not limited to pCMV and pREP vectors (obtained from Invitrogen, San Diego, CA).

Stably or transiently transfected mammalian cells may be prepared by methods that are well known in the art. Cells are transfected with an expression vector having a selectable marker gene

such as the gene for thymidine kinase, dihydrofolate reductase, or the like. Stable transfection of cells is conveniently achieved by growing the transfected cells under conditions that promote growth of cells expressing the marker gene.

5 The heterologous DNA encoding the human alpha-1E calcium channel subunit may be integrated into cell's chromosomal material or may be 10 maintained in the cell as an episomal element. Cells containing such heterologous DNA may be passaged and/or subcultured, according to methods appropriate to the cell type and known in the art.

15 B. Functional Expression of α -1E in *Xenopus* oocytes

A plasmid, such as a pBluescript SK+ plasmid, containing the full-length NX-HE1 coding sequence is used to produce complementary RNA, using an 20 appropriate RNA polymerase, such as T7 or SP6 polymerase. Such RNA is injected into oocytes from *Xenopus laevis* (about 5-10 pg/oocyte), according to methods known in the art. For 25 expression of a functional calcium channel in oocytes, coexpression of a calcium channel alpha-2 subunit, such as the rabbit skeletal muscle alpha-2 subunit (Mori et al., incorporated herein by reference) may be required. Additionally, inclusion of a calcium channel Beta subunit, such 30 as the Beta-3 subunit from rabbit heart (Hulin et al., incorporated herein by reference), helps optimize functional expression of the channel. Coding sequences for the aforementioned alpha-2 and beta-3 subunits have been published as cited 35 above, and construction of expression vectors suitable for injection into oocytes is within the skill of the practitioner. For expression in

oocytes, approximately equimolar amounts of RNA are injected into each oocyte.

5 c. Functional Expression of α -1E in Mammalian Cells

10 One cell type that is particularly amenable to transient or stable transfection is the human embryonic kidney cell line HEK 293 (ATCC Accession No. CRL1573). Such cells can be transiently cotransfected with the α -1E subunit cDNA expression plasmid, and one or more of an α_2 , calcium channel subunit cDNA expression plasmid, an β_1 subunit cDNA expression plasmid as detailed in Example 4 (Williams).

15 Stable transfection can also be achieved in HEK 293 cells according to standard methods (Ausubel). Suitable vectors for stable transfection include pcDNA1 and pcDNA3.

20 Transfected cells are selected, for example by differential growth in limiting medium, according to methods known in the art. Such cells are subcloned and tested, for example by Northern blot analysis, for the evidence for the expression of the human alpha-1E calcium channel.

25 Alternatively or in addition, individual transfected cells can be analyzed electrophysiologically for the presence of voltage-activated calcium currents (using barium as carrier, as described in Section IV. Such 30 cells are useful in functional and/or binding assays, as described in Section V.

IV. Electrophysiological Measurements

35 A. Recording of Calcium currents in *Xenopus* oocytes

Functional expression of a heterologous α -1E calcium channel subunit can be measured in *Xenopus* oocytes injected with heterologous RNA, as

described in Section III. Currents are conveniently recorded using a standard two-microelectrode voltage-clamp connected to an amplification system. Cells are placed in a chloride-free bathing solution containing about 40 mM Ba(OH)₂, such that barium acts as current carrier in the system. Channels are activated by periodic delivery of voltage pulses. Currents are measured and normalized according to procedures known to those familiar with the art pertaining to electrophysiology.

V. Utility

The present invention includes methods for identifying therapeutic compounds, such as calcium channel agonist and antagonists, that modulate the activity of calcium channels. Such assays are useful as screens for new therapeutic compounds having calcium channel agonist and/or antagonist activity. Presently, calcium channel antagonists directed at L-type calcium channels find use in the treatment of hypertension, subarachnoid hemorrhage and variant forms of angina. Certain N-type calcium channel antagonists have been found to be useful in reducing cerebral ischemia, as described in co-owned U.S. Patents 4,051,403 and 5,189,020, incorporated herein by reference.

According to the present invention, eukaryotic cells expressing heterologous α -1E calcium channel subunits encoded by heterologous DNA as described herein are useful for screening for α -1E subtype-specific compounds, and for predicting the relative potencies of such compounds.

In particular, cells expressing such heterologous calcium channels of the α -1E subtype can be used in binding assays, wherein whole cells

or membranes thereof are tested for ability to bind a test compound, generally by measuring the ability of the test compound to displace a known ligand of the channel. One appropriate functional 5 ligand which binds to the channel and blocks α -1E calcium currents is the spider toxin Aga-IIIA (isolated as described by Mintz, et al., incorporated herein by reference).

Methods for carrying out such screening 10 assays are well known in the art, and setting up such assays is within the skill of the practitioner. Co-owned allowed U.S. patent application 07/855,269, incorporated herein by reference, describes assays which utilize isolated neuronal 15 calcium channels for screening of certain types of N-type calcium channel antagonists. Such methods are adaptable to the present invention.

Alternatively, or additionally, such screening assays may include assays in which is 20 determined the functional activity of an expressed calcium channel. In such an assay, drugs which alter such activity, such as calcium current activity, are candidates for calcium channel-based therapeutics, of the types suggested above or or 25 additional types.

The following examples illustrate, but in no way are intended to limit the present invention.

30

Materials and Methods

A lambda-ZAP cDNA human hippocampal cDNA library was obtained from Stratagene (La Jolla, CA). A eukaryotic expression vector pcDNA III was obtained from Invitrogen (San Diego CA). T4 DNA 35 ligase and T4 DNA polymerase were obtained from New England Biolabs (Beverly, MA); Nitrocellulose

filters were obtained from Schleicher and Schuell (Keene, NH).

Bluescript SK+(BS) was obtained from Stratagene (San Diego, CA).

5 Dephosphorylated Calf intestinal phosphatase (CIP) was obtained from New England Biolabs (Beverly, MA).

10 Synthetic oligonucleotide linkers and primers were prepared using commercially available automated oligonucleotide synthesizers, such as obtained from Applied Biosystems (Foster City, CA). Alternatively, custom designed synthetic oligonucleotides may be purchased, for example, from Synthetic Genetics (San Diego, CA). cDNA 15 synthesis kit and random priming labeling kits were obtained from Boehringer-Mannheim Biochemical (BMB, Indianapolis, IN).

20 Standard molecular biology and cloning techniques were performed essentially as previously described in Ausubel, et al., Sambrook, et al., and Maniatis, et al.

Example 1

Isolation of partial cDNA clones

25 A. Clone H6

One million recombinants of a λ ZAP II (Stratagene, La Jolla, CA) human hippocampal cDNA library were screened in duplicate at a density of 50,000 plaques per 150 mm plate using two 30 radiolabeled 1.6 kb (Hind III and Xho I digested) fragments of the rat Class B $\alpha 1$ subunit cDNA (for the sequence of the rat Class B $\alpha 1$ subunit see Dubel et al. (1992) Proc. Natl. Acad. Sci. 89:5058-5062, incorporated herein by reference):

Fragment	Nucleotides
HindIII-HindIII	712 to 2288
Xhol-Xhol	3793-5394

5 The hybridization was performed under standard conditions (5x SSPE, 5X Denhardt's, 0.1% SDS, 1 mg/ml salmon sperm DNA; recipes found in Sambrook et al. (1989) Molecular Cloning, A 10 Laboratory Manual, Cold Spring Harbor Laboratory Press). Filters were washed under medium stringency conditions (0.2X SSPE, 0.1% SDS, 50°C). H6 was one of two Class E specific clones 15 isolated. H6 bacteriophage was plaque purified using standard methods (J. Sambrook et al. (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press), double stranded BS plasmid cDNA was isolated using standard 20 phagemid rescue procedures (Stratagene), and DNA sequence was obtained using PCR based fluorescence dye termintaor procedures (Applied Biosystems).

B. Clone H24

25 One million recombinants of a λZAP II (Stratagene) human hippocampal cDNA library were screened in duplicate at a density of 50,000 plaques per 150 mm plate using a radiolabeled NotI/EcoRI fragment (nucleotides 118 to 705) of the rat Class B α1 subunit cDNA (for the sequence 30 of the rat Class B α1 subunit see Dubel et al. (1992) Proc. Natl. Acad. Sci. 89:5058-5062). The hybridization was performed under standard conditions. Filters were washed under medium stringency conditions H24 was one of 5 positives 35 isolated in the screen (4 of which were Class E clones). H24 bacteriophage was plaque purified,

double standard plasmid cDNA was prepared, and the clone was characterized by DNA sequencing.

C. Clone I2

5 One million recombinants of a λ ZAP II (Stratagene) human hippocampal cDNA library were screened in duplicate at a density of 50,000 plaques per 150 mm plate using a radiolabeled PstI/ ApaI fragment (nucleotides 4194 to 4740) from the cDNA fragment H6. The hybridization was 10 performed under standard conditions. Filters were washed under medium stringency conditions. I2 was one of 13 positives isolated in the screen. I2 bacteriophage was plaque purified, double standard 15 plasmid cDNA was prepared, and the clone was characterized by DNA sequencing.

D. Clone 3-69A

One million recombinants of a λ ZAP II (Stratagene) human hippocampal cDNA library were 20 screened in duplicate at a density of 50,000 plaques per 150 mm plate using a radiolabeled DNA oligonucleotide probe derived from a rat neuronal alpha subunit cDNA. The hybridization was 25 performed under standard conditions. Filters were washed under medium stringency conditions. 3-69A was one of 37 positives isolated in the screen. 3-69A bacteriophage was plaque purified, double standard 30 plasmid cDNA was prepared, and the clone was characterized by DNA sequencing.

Example 2

construction of full length Human α 1E cDNA

α -1E cDNA clones H24, H6, I2, and 3-69A 35 overlap and include the nucleotide sequence which codes for the entire α -1E open reading frame, nt 107 to 6912 (SEQ ID No: 1). Restriction

fragments of these partial cDNA clones were ligated together to generate a full-length α -1E cDNA in a eukaryotic expression vector (pcDNA III-Invitrogen). The resulting construct was named

5 NX-HE1. The construction of NX-HE1 was performed in multiple steps as described in detail below: 1) recloning of H24 into Bluescript SK+(BS) to obtain proper orientation (Construct 1), 2) ligation of an XhoI fragment (nt 1 to 1374) from Construct 1

10 into pcDNA III (to form Construct 2), 3) ligation of an XhoI/ApaI fragment from H6 (nt 1374 to 4181) into Construct 2 (to form Construct 3), 4) ligation of an SfiI/BamHI fragment from 3-69A (nt 5884 to 3' untranslated) into I2 (to form

15 Construct 4), and 5) ligation of an ApaI fragment (nt 4181 to 3' untranslated into Construct 3 to from the full length clone SEQ ID No.1).

1) To obtain Construct 1, H24 was digested with EcoRI and religated into EcoRI digested BS.

20 The purpose of this ligation was to reorient the original H24 clone within the BS polylinker such that the BS XhoI site was 5' relative to the coding region.

25 2) Construct 2 was digested with XhoI and the resultant 1.4 kb fragment was purified from an agarose gel and ligated into XhoI digested pcDNAIII dephosphorylated with calf intestinal phosphatase (CIP). This construct thus contained the 5' end of the clone (nt 1 to 1374).

30 3) H6 was then digested with XhoI and Apa I and the resultant 2.8 kb fragment (nt 1374 to 4181) was ligated into XhoI /ApaI digested and CIP treated Construct 2. The ligated product was referred to as Construct 3.

35 4) The remainder of the 3' end was constructed separately. First, 3-69A was digested with SfiI and BamHI to yield a 2.7 kb fragment (nt

5884 to 3' untranslated), which was gel purified and ligated into SfiI/BamHI digested and CIP treated I2. The ligated product was referred to as Construct 4. (All ligation junctions were sequenced prior to proceeding with the next step).

5 5) The final construct was prepared by digesting Construct 4 with ApaI, yielding a 4.1 kb fragment (nt 4182 to 3' ut) which was gel purified and ligated into ApaI cut CIP treated 10 Construct 3. This ligated product containing the full length sequence was named NX-HE1.

Example 3

Functional Expression of α -1E in Xenopus oocytes

15 The α -1E full-length DNA sequence SEQ ID NO:1 was subcloned into pBluescript SK + to obtain plasmid ph α -1E(SK+). Complementary RNA was synthesized from the plasmid *in vitro* using T7 or SP6 polymerase. Additionally, complementary RNA 20 is synthesized from plasmids containing calcium channel alpha-2 subunits and Beta subunits known in the art (Mori, Williams, Tanabe). RNA from each subunit was injected in equimolar ratios using 0.1 μ g ml $^{-1}$ α_1 , 0.1 μ g ml $^{-1}$ α_2 , and 0.03 μ g ml $^{-1}$ β ; ~ 50 nl is injected per cell. The α -1E currents were recorded by a standard two- 25 microelectrode voltage-clamp using a Warner amplifier (OC-725A) in a chloride free bath solution, containing (in mM): Ba(OH) $_2$, 5; tetraethylammonium-OH, 40; KOH, 2; HEPES, 5; pH 30 7.4 adjusted with methanesulphonic acid. Data were sampled at 10 kHz and filtered at 2 kHz. Leak and capacitance currents are subtracted off-line by a P/4 protocol. Voltage pulses are 35 delivered every 15 s. For steady-state inactivation experiments, voltage pulses were delivered every 20 s. Peak normalized currents

were fitted to a Hodgkin-Huxley inactivation curve. Figure 2 shows a trace of a barium current measured in a *Xenopus* oocyte previously injected with mRNA encoding the human calcium channel 5 subunit α -1E (SEQ ID NO:2) plus RNA encoding alpha-2 and beta subunits, as described above. The current was invoked from a holding potential of -80 mV to a test potential of 0mV.

The spider toxin AgaIIIA was effective to 10 block this current, at a batch concentration of 50 nM. Other calcium channel blocking compounds which were unable to block this current and maximum concentrations tested are as follows: omega conotoxins MVIIA (5 μ M), MVIIC (5 μ M), TVIA 15 (5 μ M), Aga IVA (100 nM) and dehydropyridines.

Example 4

Expression of α -1E in Mammalian Cells

Human embryonic kidney cells (HEK 293 20 cells) were transiently and stably transfected with human neuronal DNA encoding calcium channel subunits.

A. Transfection of HEK 293 Cells

25 Separate expression vectors containing DNA encoding human neuronal calcium channel α -1E, rat α_2 , and β_1 subunits, were used. constructed as described in Example 2. The α -1E coding sequence described herein as SEQ ID NO: 1 was incorporated 30 into a pcDNA1+3 vector, as described above, with addition of a c-myc epitope tag on the 5' end of the α -1E coding sequence, for monitoring, according to established methods. The vector was used for stable transfection of HEK 293 cells 35 using the calcium phosphate transfection procedure (Ausubel). Culture plates containing about two million HEK 293 cells, were transfected with 1 ml

of DNA/calcium phosphate precipitate containing 5 µg of each of the vectors. After 10-20 days of growth in media containing 500 µg G418, colonies had formed and were isolated according to standard 5 procedures.

Expression of α -1E subunit by cells was monitored by fluorescence immunolabeling. Cells were incubated with fluorescently tagged antibodies (from commercial sources) directed to the c-myc epitope, the coding sequence for which was added 10 to the 5' end of the α -1E coding sequence. Expression of the α -1E subunit having the epitope was evidenced by immunofluorescence.

15 While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the 20 invention.

IT IS CLAIMED:

1. An isolated DNA fragment, comprising a sequence of nucleotides that encodes an alpha-1E 5 subunit of a human neuronal calcium channel, and having the sequence presented as SEQ ID NO:1.
2. An alpha-1E polypeptide subunit of a human neuronal calcium channel having the sequence 10 presented as SEQ ID NO:2.
3. A mammalian expression vector containing the nucleotide sequence presented as SEQ ID NO:1.
- 15 4. A eukaryotic cell, which includes a heterologous DNA having the sequence presented as SEQ ID NO:1.
- 20 5. The cell of claim 4, wherein the cell is a Xenopus oocyte.
6. The cell of claim 4, wherein the cell is a human embryonic kidney cell.
- 25 7. The cell of claim 4, wherein said DNA expresses a neuronal calcium channel including a polypeptide subunit having the sequence presented as SEQ ID NO: 2.
- 30 8. The cell line of claim 7, which further expresses neuronal calcium channel subunits α_1 and β .
- 35 9. A method of screening a compound capable of blocking calcium uptake in human neuronal cells, comprising

exposing the cell of claim 7 with the test compound,

examining the effect of said exposing on calcium uptake into the cells, and

5 selecting the compound if said exposing inhibits calcium uptake into the cells.

10. The method of claim 9, wherein said exposing includes contacting the cells with the
10 cell of claim 8.

1	GAA	TTC	CGG	CTC	TGA	GTC	TCC	CTG	TGT	CTT	GCT	TCT	GTC	TGT	TGC	TGT	GTC	TGT	GTC	48
1	E	F	R	L	*	V	S	C	L	S	A	C	C	C	C	C	C	C	V	16
49	CGG	GTG	TTC	GGC	CGC	GAT	CAC	CTT	TGT	GTG	TCT	GTC	TGT	TTA	AAC	GGG	TCC	TCC	49	
17	R	V	F	G	R	D	H	L	C	V	S	S	V	C	L	N	R	P	G	32
97	CTC	AGG	ATG	GCT	CGC	TTC	GGG	GAG	GGG	GTG	GTC	GCC	AGG	CCA	GGG	TCC	TCC	TCC	144	
33	L	R	M	A	R	F	G	E	A	V	V	A	R	P	G	S	P	V	48	
145	GGC	GAT	GGA	GAC	TGG	GAC	CAG	AGC	AGG	AAC	CGG	CAA	GGG	ACC	CCC	GTG	192			
49	G	D	G	S	D	Q	S	R	N	R	Q	G	T	P	V	V	P	V	64	
193	CCG	GCC	TGG	GGG	CAG	GGC	GGC	TAC	AAG	CAG	ACG	AAA	GCA	CAG	AGG	240				
65	P	A	S	G	Q	A	A	Y	K	Q	T	K	A	Q	R	80				
241	GCG	CGG	ACT	ATG	GCT	TTG	TAC	AAC	CCC	ATT	CCC	GTC	CGG	CAG	AAC	TGT	288			
81	A	R	T	M	A	L	Y	N	P	I	P	V	R	Q	N	C	96			
289	TTC	ACC	GTC	AAC	AGA	TCC	CTG	TTC	ATC	TTC	GGA	GAA	GAT	AAC	ATT	GTC	336			
97	F	T	V	N	R	S	L	F	I	F	G	E	D	N	I	V	112			
337	AGG	AAA	TAT	GCC	AAG	AAG	CTC	ATC	GAT	TGG	CCG	CCA	TTT	GAG	TAC	ATG	384			
113	R	K	Y	A	K	K	L	I	D	W	P	P	F	E	Y	M	128			
385	ATC	CTG	GCC	ACC	ATC	ATT	GCC	AAC	TGC	ATC	GTC	GCC	CTG	GAG	CAG	432				
129	I	L	A	T	I	I	A	N	C	I	V	L	A	L	E	Q	144			
433	CAT	CTT	CCT	GAG	GAT	GAC	AAG	ACC	CCC	ATG	TCC	CGA	AGA	CTG	GAG	AAG	480			
145	H	L	P	E	D	D	K	T	P	M	S	R	R	L	E	K	160			
481	ACA	GAA	CCT	TAT	TTC	ATT	GGG	ATC	TTT	TGC	TTT	GAA	GCT	GGG	ATC	AAA	528			
161	T	E	P	Y	F	I	G	I	F	C	F	E	A	G	I	K	176			
529	ATT	GTG	GCC	CTG	GGG	TTC	ATC	TTC	CAT	AAG	GGC	TCT	TAC	CTC	CGC	AAT	576			
177	I	V	A	L	G	F	I	F	H	K	Q	S	Y	L	R	N	192			

Fig. 1

**Fig. 1
(1 con't)**

577	GGC	TGG	AAT	GTC	ATG	GAC	TTC	ATC	GTG	GTC	CTC	AGT	GGC	ATC	CTG	GGC	624
193	G	W	N	V	M	D	F	I	V	V	L	S	G	I	L	A	208
625	ACT	GCA	GGA	ACC	CAC	TTC	AAT	ACT	CAC	GTG	GAC	CTG	AGG	ACC	CTC	CGG	672
209	T	A	G	T	H	F	N	T	H	V	D	L	R	T	L	R	224
673	GCT	GTG	CGT	GTC	CTG	CGG	CCT	TTG	AAG	CTC	GTG	TCA	GGG	ATA	CCT	AGC	720
225	A	V	R	V	L	R	P	L	K	L	V	S	G	I	P	S	240
721	CTG	CAG	ATT	GTG	TTC	AAG	TCC	ATC	ATG	AAG	GCC	ATG	GTG	CCT	CTT	CTG	768
241	L	Q	I	V	L	K	S	I	M	K	A	M	V	P	L	L	256
769	CAG	ATT	GGC	CTT	CTG	CTG	TTC	TTT	GCC	ATC	CTG	ATG	TTT	GCT	ATC	ATT	816
257	Q	T	G	L	L	L	F	F	A	I	L	M	F	A	I	I	272
817	GGT	TTG	GAG	TTC	TAC	AGT	GGC	AAG	TTA	CAT	CGA	GCG	TGC	TTC	ATG	AAC	864
273	G	L	E	F	Y	S	G	K	L	H	R	A	C	F	M	N	288
865	AAT	TCA	GGT	ATT	CTA	GAA	GGA	TTT	GAC	CCC	CCT	CAC	CCA	TGT	GGT	GTG	912
289	N	S	G	I	L	E	G	F	D	P	P	H	P	C	G	V	304
913	CAG	GGC	TGC	CCA	GCT	GGT	TAT	GAA	TGC	AAG	GAC	TGG	ATC	GGC	CCC	AAT	960
305	Q	G	C	P	A	G	Y	E	C	K	D	W	I	G	P	N	320
961	GAT	GGG	ATC	ACC	CAG	TTT	GAT	AAC	ATC	CTT	TTT	GCT	GTG	CTG	ACT	GTC	1008
321	D	G	I	T	Q	F	D	N	I	L	F	A	V	L	T	V	336
1009	TTC	CAG	TGC	ATC	ACC	ATG	GAA	GGG	TGG	ACC	ACT	GTG	CTG	TAC	AAT	ACC	1056
337	F	Q	C	I	T	M	E	G	W	T	T	V	L	Y	N	T	352
1057	AAT	GAT	GCC	TTA	GGA	GCC	ACC	TGG	AAT	TGG	CTG	TAC	TTC	ATC	CCC	CTC	1104
353	N	D	A	L	G	A	T	W	N	W	L	Y	F	I	P	L	368
1105	ATC	ATC	ATT	GGA	TCC	TTC	TTT	GTT	CTC	AAC	CTA	GTC	CTG	GGG	~T3	CTT	1152
369	I	I	I	G	S	F	F	V	L	N	L	V	L	G	V	L	384
1153	TCC	GGG	GAA	TTT	GCC	AAA	GAG	AGA	GAG	GAG	AAC	CGA	AGG	GCT			1200
385	S	G	E	F	A	K	E	R	E	R	V	E	N	R	R	A	400

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Fig. 1
(2 con't)

1201	TTC	ATG	AAG	CTG	CGG	CGC	CAG	CAG	ATT	GAG	CGT	CTG	AAT	GGC	1248
401	F	M	K	L	R	Q	Q	Q	I	E	R	L	N	G	416
1249	TAC	CGT	GGC	TGG	ATA	GAC	AAA	GCA	GAG	GAA	GTC	ATG	CTC	GCT	1296
417	Y	R	A	W	I	D	K	A	E	E	V	M	L	A	432
1297	AAT	AAA	AAT	GCT	CGG	ACA	TCC	GCC	TTA	GAA	GTG	CTT	CGA	AGG	1344
433	N	K	N	A	G	T	S	A	L	E	V	R	R	A	448
1345	ATC	AAG	AGG	AGC	CGG	ACA	GAG	GCC	ATG	ACT	CGA	GAC	TCC	AGT	1392
449	I	K	R	S	R	T	E	A	M	T	R	D	S	D	464
1393	CAC	TGT	GTT	GAT	ATC	TCC	TCT	GTG	GGC	ACA	CCT	CTG	GCC	CGA	1440
465	H	C	V	D	I	S	S	V	G	T	P	L	A	R	480
1441	ATC	AAA	AGT	GCA	AAG	GTA	GAC	GGG	GTC	TCT	TAT	TTC	CGG	CAC	1488
481	I	K	S	A	K	V	D	G	V	S	Y	F	R	H	496
1489	AGG	CTT	CTG	CGC	ATC	TCC	ATT	CGC	CAC	ATG	GTT	AAA	TCC	CAG	1536
497	R	L	L	R	I	S	I	R	H	M	V	K	S	Q	512
1537	TAC	TGG	ATT	CTG	CTG	AGG	CCT	GTG	GCA	CTC	AAC	ACT	GCC	TGT	1584
513	Y	W	I	V	L	S	L	V	A	L	N	T	A	C	528
1585	ATT	GTC	CAT	CAC	AAC	CAG	CCC	CAG	TGG	CTC	ACC	CTC	CTC	TAC	1632
529	I	V	H	H	N	Q	P	Q	W	L	T	H	L	Y	544
1633	GCA	GAA	TTT	CTG	TTT	CTG	GGG	CTC	TTC	CTC	TTC	GAG	ATG	TCC	1680
545	A	E	F	L	F	L	G	L	F	L	L	E	M	S	560
1681	ATG	TAT	GGC	ATG	GGG	CCT	CGC	CTT	TAT	TTT	CAC	TCT	TCA	TTC	1728
561	M	Y	G	M	G	P	R	L	Y	F	H	S	F	N	576
1729	TTT	GAT	TTT	GGG	GTC	ACA	GTG	GGC	AGT	ATC	TTT	GAA	GTG	TGC	1776
577	F	D	F	G	V	T	V	G	S	I	F	E	V	W	592
1777	ATC	TTC	AGA	CCT	GGT	ACG	TCT	TTT	GGA	ATC	AGT	GTC	TTG	CGA	1824
593	I	F	R	P	G	T	S	F	G	I	S	V	L	A	608

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Fig. 1
(3 con't)

1825	CGG	CTT	CTA	AGA	ATA	TTT	AAA	ATA	ACC	AAG	TAT	TGG	GCT	TCC	CTA	CGG	1872	
609	R	L	L	R	I	F	K	I	T	K	Y	W	A	S	L	R	624	
1873	AAT	TTC	GTC	GTC	TCC	TTG	ATG	AGC	TCA	ATG	AAG	TCT	ATC	ATC	AGT	TTG	1920	
625	N	L	V	V	S	L	M	S	S	M	K	S	I	I	S	L	640	
1921	CTT	TTC	CTC	CTC	TTC	CTC	ATC	GTT	GTC	TTT	GCT	CTC	CTA	GGA	ATG	1968		
641	L	F	L	F	L	F	I	V	V	F	A	L	L	G	M	656		
1969	CAG	TTA	TTT	GGA	GGC	GGC	AGG	TTT	AAC	TTT	AAT	GAT	GGG	ACT	CCT	TCG	GCA	2016
657	Q	L	F	G	G	R	F	N	F	N	D	G	T	P	S	A	672	
2017	AAT	TTT	GAT	ACC	TTC	CCT	GCA	GCC	ATC	ATG	ACT	GTG	TTC	CAG	AAC	CTG	2064	
673	N	F	D	T	F	P	A	A	I	M	T	V	F	Q	I	L	688	
2065	ACG	GGT	GAG	GAC	TGG	AAT	GAG	GTG	ATG	TAC	AAT	GGG	ATC	CGC	TCC	CAG	2112	
689	T	G	E	D	W	N	E	V	M	Y	N	G	I	R	S	Q	704	
2113	GGT	GGG	GTC	AGC	TCA	GGC	ATG	TGG	TCT	GCC	ATC	TAC	TTC	ATT	GTG	CTC	2160	
705	G	G	V	S	S	G	M	W	S	A	I	Y	F	I	V	L	720	
2161	ACC	TTC	TTT	GGC	AAC	TAC	ACG	CTA	CTG	AAT	GTG	TTC	TTG	GCT	ATC	GCT	2208	
721	T	L	F	G	N	Y	T	L	L	N	V	F	L	A	I	A	736	
2209	GTG	GAT	AAT	CTC	GCC	AAC	GCC	CAG	GAA	CTG	ACC	AAG	GAT	GAA	CAG	GAG	2256	
757	V	D	N	L	A	N	A	Q	E	L	T	K	D	E	Q	E	752	
2257	GAA	GAA	GAG	GCC	TTC	AAC	CAG	AAA	CAT	GCA	CTG	CAG	AAG	GCC	AAG	GAG	2304	
753	E	E	E	A	F	N	Q	K	H	A	L	Q	K	A	K	E	768	
2305	GTC	AGC	CCG	ATG	TCT	GCA	CCC	AAC	ATG	CCT	TCG	ATC	GAA	AGA	GAC	AGA	2352	
769	V	S	P	M	S	A	P	N	M	P	S	I	E	R	D	R	784	
2353	AGG	AGA	AGA	CAC	CAC	ATG	TGG	ATG	TGG	GAG	CCA	CGC	AGC	CAC	CTG	2400		
785	R	R	H	H	H	M	S	M	W	E	P	R	S	S	H	L	800	

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Fig. 1
(4 con't)

2401 801	AGG R	GAG E	CGG R	AGG R	CGG R	CAC H	CAC H	ATG M	TCC S	GTG V	GAG W	CAG E	CGT Q	ACC R	2448 816	
2449 817	AGC S	CAG Q	CTG L	AGG R	AAG K	CAC H	ATG M	CAG Q	ATG M	TCC S	AGC S	CAG Q	GCC E	CTC A	AAC L	2496 832
2497 833	AGA R	GAG E	GAG A	CCG P	CCG T	ACC M	ATG N	AAC P	CCC L	CTC N	AAC P	CTG L	CGA N	CCC P	CTC L	2544 848
2545 849	AGC S	TCC L	CTC N	AAC P	CCG L	CAC N	CTG A	AAAT H	CCC P	AGC S	CTT L	TAT Y	CGG R	CGA R	CCC P	2592 864
2593 865	AGG R	GCC A	ATG I	GAG E	GGC G	CTG L	GGC A	CTG L	GGC A	CTG L	GAG A	TTC E	GAG K	TTC F	GAG E	2640 880
2641 881	GAG E	GAG E	CGGC R	ATC I	AGC S	CGT R	GGG G	GGG G	TCC S	CTC L	AAG K	GAT G	GGG D	GAG G	GGG D	2688 896
2689 897	CGA R	TCC S	AGT S	GCC A	CTG A	GAC L	AAC D	CAG N	AGG Q	ACC R	CCT T	TTG P	TCC L	CTG S	GGC L	2736 912
2737 913	CGG R	GAG E	CCA P	TGG P	CTG W	GCC A	AGG R	CCC P	TGT C	CAT H	GGA G	AAC N	TGT C	GAC C	CCG D	2784 928
2785 929	ACT T	CAG Q	CAG E	GCA A	GGG G	GGG G	GGA G	GGA G	TCT G	GTG V	ACC V	TTT T	GAG F	GAC E	GAC D	2832 944
2833 945	CGG R	GGC A	AGG R	CAC H	AGG Q	CAG R	AGC Q	CAA R	CGG R	CGC S	AGC R	CAT H	CGC R	GTC R	GTC V	2880 960
2881 961	AGG R	ACA T	GAA E	GGC G	AAG K	GAG S	TCC S	TCT S	TCA S	GCC A	TCC S	CGG R	AGC S	TCT R	GCC A	2928 976
2929 977	AGC S	CAG Q	CGG E	AGT R	CTG S	GAT L	GAA D	GCC E	ATG A	CCC M	ACT P	GAA T	GGG G	GAG S	AAG A	2976 992
2977 993	GAC D	CAT H	GAG E	CTG L	AGG R	AAC G	GGC N	CAT H	GGT G	GCC A	AAG A	ATC P	CAA T	ATC I	CAA Q	3024 1008

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Fig. 1
(5 con't)

3025	GAA	GAG	AGA	GCC	CAG	GAT	TTA	AGG	AGG	ACC	AAC	AGT	CTG	ATG	GTG	TCC	3072
1009	E	E	R	A	Q	D	L	R	R	T	N	S	L	M	V	S	1024
3073	AGA	GGC	TCC	GGG	CTG	GCA	GGG	GGC	CTT	GAT	GAG	GCT	GAC	ACC	CCC	CTA	3120
1025	R	G	S	G	L	A	G	G	L	D	E	A	D	T	P	L	1040
3121	GTC	CTG	CCC	CAT	CCT	GAG	CTG	GAA	GTG	GGG	AAG	CAC	GTG	CTG	ACG	3168	
1041	V	L	P	H	P	E	L	E	V	G	K	H	V	V	L	T	1056
3169	GAG	CAG	GAG	CCA	GAA	GGC	AGC	AGT	GAG	CAG	GGC	CTC	CTG	GGG	AAT	GTG	3216
1057	E	Q	E	P	E	G	S	S	E	Q	A	L	L	G	N	V	1072
3217	CAG	CTA	GAC	ATG	GGC	CGC	ATC	AGC	CAG	AGC	GAG	CCT	GAC	CTC	TCC	3264	
1073	Q	L	D	M	G	R	V	I	S	Q	S	E	P	D	L	S	1088
3265	TGC	ATC	ACG	GCC	AAC	ACG	GAC	AAG	GCC	ACC	ACC	GAG	AGC	ACC	GTC	3312	
1089	C	I	T	A	N	T	D	K	A	T	T	E	S	T	S	V	1104
3313	ACC	GTC	GCC	ATC	CCC	GAC	GTG	GAC	CCC	TTG	GTG	GAC	TCA	ACC	GTG	3360	
1105	T	V	A	I	P	D	V	D	P	I,	V	D	S	T	V	V	1120
3361	CAC	ATT	AGC	AAC	AAG	ACG	GAT	GGG	GAA	GCC	AGT	CCC	TTG	AAG	GAG	GCA	3408
1121	H	I	S	N	K	T	D	G	E	A	S	P	L	K	E	A	1136
3409	GAG	ATC	AGA	GAG	GAT	GAG	GAG	GTG	GAG	AAG	AAG	CAG	AAG	AAG	AAG	3456	
1137	E	I	R	E	D	E	E	E	V	E	K	K	K	Q	K	K	1152
3457	GAG	AAG	CGT	GAG	ACA	GGA	AAA	GCC	ATG	GTG	CCC	CAC	AGC	TCA	ATG	TTC	3504
1153	E	K	R	E	T	G	K	A	M	V	P	H	S	S	M	F	1168
3505	ATC	TTC	AGC	ACC	AAC	CCG	ATC	CGG	AGG	GCC	TGC	CAC	TAC	ATC	GTG	3552	
1169	I	F	S	T	T	N	P	I	R	R	A	C	H	Y	I	V	1184
3553	AAC	CTG	CGC	TAC	TTT	GAG	ATG	TGC	ATC	CTC	CTG	GTG	ATT	GCA	GCC	AGC	3600
1185	N	L	R	Y	F	E	M	C	I	L	L	V	I	A	A	S	1200
3601	AGC	ATC	GCC	CTG	CGG	GCA	GAG	GAC	CCC	GTC	CTG	ACC	AAC	TCG	GAG	CGC	3648

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Fig. 1
(6 cont.)

1201	S	I	A	L	A	E	D	P	V	L	T	N	S	E	R	1216	
3649	AAC	AAA	GTC	CTG	AGG	TAT	TTT	GAC	TAT	GTG	TTC	ACG	GGC	GTG	TTC	ACC	3696
1217	N	K	V	L	R	Y	F	D	Y	V	F	T	G	V	F	T	1232
3697	TTT	GAG	ATG	GTT	ATA	AAG	ATG	ATA	GAC	CAA	GGC	TTG	ATC	CTG	CAG	GAT	3744
1233	F	E	M	V	I	K	M	I	D	Q	G	L	I	L	Q	D	1248
3745	GGG	TCC	TAC	TTC	CGA	GAC	TTC	TGG	AAC	ATC	CTG	GAC	TTT	GTG	GTC	GTC	3792
1249	G	S	Y	F	R	D	L	W	N	I	L	D	F	V	V	V	1264
3793	GTT	GGC	GCA	TTC	GTG	GCC	TTT	GCT	CTG	GCG	AAC	GCT	TTG	GGA	ACC	AAC	3840
1265	V	G	A	L	V	A	F	A	L	A	N	A	L	G	T	N	1280
3841	AAA	GGA	CGG	GAC	ATC	AAG	ACC	ATC	AAG	TCT	CTG	CGG	GTG	CTC	CGA	GTT	3888
1281	K	G	R	D	I	K	T	I	K	S	L	R	V	L	R	V	1296
3889	CTA	AGG	CCA	CTG	AAA	ACC	ATC	AAG	CGC	TTG	CCC	AAG	CTC	AAG	GCC	GTC	3936
1297	L	R	P	L	K	T	I	K	R	L	P	K	L	K	A	V	1312
3937	TTC	GAC	TGC	GTA	GTG	ACC	TCC	TTG	AAG	AAT	GTC	TTC	AAC	ATA	CTC	ATT	3984
1313	F	D	C	V	V	T	S	L	K	N	V	F	N	I	L	I	1328
3985	GTG	TAC	AAG	CTC	TTC	ATG	TTC	ATC	TTT	GCT	GTC	ATC	GCA	GTT	CAG	CTC	4032
1329	V	Y	K	L	F	M	F	I	F	A	V	I	A	V	Q	L	1344
4033	TTC	AAG	GCA	AAG	TTC	TTT	TAT	TGC	ACG	GAC	AGT	TCC	AAG	GAC	ACA	GAG	4080
1345	F	K	G	K	F	F	Y	C	T	D	S	S	K	D	T	E	1360
4081	AAG	GAG	TGC	ATA	GGC	AAC	TAT	GTA	GAT	CAT	GAG	AAA	AAC	AGG	ATG	GAG	4128
1361	K	E	C	I	G	N	Y	V	D	H	E	K	N	K	M	E	1376
4129	GTG	AAG	GGC	CGG	GAA	TGG	AAG	CGC	CAT	GAA	TTC	CAC	TAC	GAC	AAC	ATT	4176
1377	V	K	G	R	E	W	K	R	H	E	F	H	Y	D	N	I	1392
4177	ATC	TGG	GCC	CTG	CTG	ACC	CTC	TTC	ACC	GTC	TCC	ACA	GGG	GAA	TGG	4224	
1393	I	W	A	L	L	T	L	F	T	V	S	T	G	E	G	W	1408

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Fig. 1
(7 con't)

4225	CCT CAA GTT CTG CAG CAC TCT GTA GAT GTG ACA GAG GAA GAC CGA GGC	4272
1409	P Q V L Q H S V D V T E E D R G	1424
4273	CCA AGC CGC AGC AAC CGC ATG GAG ATG TCT ATC TTT TAT GTA GTC TAC	4320
1425	P S R S N R M E M S I F Y V V	1440
4321	TTT GTG GTC TTC CCC TTC TTT GTC AAT ATC TTT GTG GCT CTC ATC	4368
1441	F V F P F F V N I F V A L I	1456
4369	ATC ATC ACC TTC CAG GAG CAA GGG GAT AAG ATG ATG GAG TGC AGC	4416
1457	I I T F Q E Q G D K M M E E C S	1472
4417	CTG GAG AAG AAT GAG AGG GCG TGC ATC GAC TTC GCA ATC AGC GCC CAA	4464
1473	L E K N E R A C I D F A I S A Q	1488
4465	CCT CTC ACC CGC TAC ATG CCG CAG AAC AGA CAC ACC TTC CAG TAC CGC	4512
1489	P L T R Y M P Q N R H T F Q Y R	1504
4513	GTG TGG CAC TTT GTG GTG TCT CCG TCC TTT GAG TAC ACC ATT ATG GCC	4560
1505	V W H F V V S P S F E Y T I M A	1520
4561	ATG ATC GCC TTG AAT ACT GTT GTG CTG ATG ATG AAG TAT TAT TCT GCT	4608
1521	M I A L N T V V L M M K Y Y S A	1536
4609	CCC TGT ACC TAT GAG CTG GCC CTG AAG TAC CTG AAT ATC GCC TTC ACC	4656
1537	P C T Y E L A L K Y L N I A F T	1552
4657	ATG GTG TTT TCC CTG GAA TGT GTC CTG AAG GTC ATC GCT TTT GGC TTT	4704
1553	M V F S L E C V L K V I A F G F	1568
4705	TTG AAC TAT TTC CGA GAC ACC TGG AAT ATC TTT GAC TTC ATC ACC GTG	4752
1569	L N Y F R D T W N I F D F I T V	1584
4753	ATT GGC AGT ATC ACA GAA ATT ATC CTG ACA GAC AGC AAG CTG GTG AAC	4800
1585	I G S I T E I I L T D S K L V N	1600
4801	ACC AGT GGC TTC ATT ATG AGC TTT CTG AAG CTC TTC CGA GCT GCC CGC	4848
1601	T S G F N M S F L K R A A R	1616

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Fig. 1
(8 con't)

4849	CTC	ATA	AAG	CTC	CTG	CGT	CAG	GGC	TAT	ACC	ATA	CGC	ATT	TTG	CTG	TGG	4896
1617	L	I	K	L	L	R	Q	G	Y	T	I	R	I	L	L	W	1632
4897	ACC	TTT	GTG	CAG	TCC	TTT	AAG	GCC	CTC	CCT	TAT	GTC	TGC	CTT	TTA	ATT	4944
1633	T	F	V	Q	S	F	K	A	L	P	Y	V	C	L	L	I	1648
4945	GCC	ATG	CTT	TTC	TTC	ATT	TAT	GCC	ATC	ATT	GGG	ATG	CAG	GTA	TTT	GGA	4992
1649	A	M	L	F	F	I	Y	A	I	I	G	M	Q	V	F	G	1664
4993	AAC	ATA	AAA	TTA	GAC	GAG	GAG	AGT	CAC	ATC	AAC	CGG	CAC	AAC	AAC	TTC	5040
1665	N	I	K	L	D	E	E	S	H	I	N	R	H	N	N	F	1680
5041	CGG	AGT	TTC	TTT	GGG	TCC	CTA	CTG	CTA	CTC	TTC	AGG	AGT	GCC	ACA	GGT	5088
1681	R	S	F	F	G	S	L	M	L	L	F	R	S	A	T	G	1696
5089	GAG	GCC	TGG	CAG	GAG	ATT	ATG	CTG	TCA	TGC	CTT	GGG	GAG	AAG	GGC	TGT	5136
1697	E	A	W	Q	E	I	M	L	S	C	L	G	E	K	G	C	1712
5137	GAG	CCT	GAC	ACC	GCA	CCA	TCA	GGG	CAG	AAC	GAG	AAC	GAA	CGC	TGC	5184	
1713	E	P	D	T	T	A	P	S	G	Q	N	E	N	E	R	C	1728
5185	GGC	ACC	GAT	CTG	GCC	TAC	GTG	TAC	TTT	GTC	TCC	TTC	ATC	TTC	TTC	TGC	5232
1729	G	T	D	L	A	Y	V	F	V	S	F	I	F	F	F	C	1744
5233	TCC	TTC	TTG	ATG	CTC	AAC	CTG	TTT	GTG	GCC	GTC	ATC	ATG	GAAC	AAC	TTT	5280
1745	S	F	L	M	L	N	L	F	V	A	V	I	M	D	N	F	1760
5281	GAG	TAC	CTG	ACT	CGG	GAC	TCC	TCC	ATC	CTG	GGG	CCT	CAC	CAC	TTG	GAC	5328
1761	E	Y	L	T	R	D	S	S	I	L	G	P	H	H	L	D	1776
5329	GAG	TTT	GTC	CGC	GTC	TGG	GCA	GAA	TAT	GAC	CGA	GCA	TGT	GGC	CGC	5376	
1777	E	F	V	R	V	W	A	E	Y	D	R	A	A	C	G	R	1792
5377	ATC	CAT	TAC	ACT	GAG	ATG	TAT	GAA	ATG	CTG	ACT	CTC	ATG	TCA	CCT	CCG	5424
1793	I	H	Y	T	E	M	Y	E	M	L	T	L	M	S	P	P	1808
5425	CTA	GGC	CTC	GGC	AAG	AGA	TGT	CCC	TCC	AAA	GTG	GCA	TAT	AAG	AGG	TTG	5472
1809	L	G	L	G	K	R	C	P	S	K	V	A	Y	K	R	L	1824

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Fig. 1
(9 con't)

5473	GTC	CTG	ATG	AAC	ATG	CCA	GTA	GCT	GAG	GAC	ATG	ACG	GTC	CAC	TTC	ACC	5520
1825	V	L	M	N	M	P	V	A	E	D	M	T	V	H	F	T	1840
5521	TCC	ACA	CTT	ATG	GCT	CTG	ATC	CGG	ACA	GCT	CTG	GAC	ATT	AAA	ATT	GCC	5568
1841	S	T	L	M	A	L	I	R	T	A	L	D	I	K	I	A	1856
5569	AAA	GCT	GCA	GAC	AGG	CAG	CAG	CTA	GAC	TCA	GAG	CTA	CAA	AAG	GAG	5616	
1857	K	G	G	A	D	R	2	Q	L	D	S	E	L	Q	K	E	1872
5617	ACC	CTA	GCC	ATC	TGG	CCT	CAC	CTA	TCC	CAG	AAG	ATG	CTG	GAT	CTG	CTT	5664
1873	T	L	A	I	W	P	H	L	S	Q	K	M	L	D	L	L	1888
5665	GTG	CCC	ATG	CCC	AAA	GCC	TCT	GAC	CTG	ACT	GTG	GGC	AAA	ATC	TAT	GCA	5712
1889	V	P	M	P	K	A	S	D	L	T	V	G	K	I	Y	A	1904
5713	GCA	ATG	ATG	ATC	ATG	GAC	TAC	TAT	AAG	CAG	AGT	AAG	GTG	AAG	AAG	CAG	5760
1905	A	M	M	I	M	D	Y	Y	K	Q	S	K	V	K	K	Q	1920
5761	AGG	CAG	CAG	CTG	GAG	GAA	CAG	AAA	AAA	GCC	CCC	ATG	TTC	CAG	CGC	ATG	5808
1921	R	Q	Q	L	E	E	Q	K	N	A	P	M	F	Q	R	M	1936
5809	GAG	CCT	TCA	TCT	CTG	CCT	CAG	GAG	ATC	ATT	GCT	AAT	GCC	AAA	GCC	CTG	5856
1937	E	P	S	S	L	P	Q	E	I	I	A	N	A	K	A	L	1952
5857	CCT	TAC	CTC	CAG	CAG	GAC	CCC	GTC	TCA	GCC	CTG	AGT	GGC	CGG	AGT	GGA	5904
1953	P	Y	L	Q	Q	D	P	V	S	G	L	S	G	P	S	G	1968
5905	TAC	CCT	TCG	ATG	AGT	CCA	CTC	TCT	CCC	CAG	GAT	ATA	TTC	CAG	TTG	GCT	5952
1969	Y	P	S	M	S	P	L	S	P	Q	D	I	F	Q	L	A	1984
5953	TGT	ATG	GAC	CCC	ACC	GAT	GAC	GGA	CAG	TTC	CAA	GAA	CGG	CAG	TCT	CTG	6000
1985	C	M	D	P	T	D	D	G	Q	F	Q	E	R	Q	S	L	2000
6001	GTG	GTG	ACA	GAC	CCT	AGC	TCC	ATG	AGA	CGT	TCA	TTT	TCC	ACT	ATT	CGG	6048
2001	V	V	T	D	P	S	S	M	R	R	S	F	S	T	I	R	2016
6049	GAT	AAG	CGT	TCA	AAT	TCC	TCG	TGG	TGG	GAG	GAA	TTC	TCC	ATG	GAG	CGA	6096
2017	D	K	R	S	N	S	S	W	L	E	E	F	S	M	E	R	2032

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**Fig. 1
(10 cont'd)**

6097	AGC	AGT	GAA	AAT	ACC	TAC	AAG	TCC	CGT	CGC	CGG	AGT	TAC	CAC	TCC	TCC		6144
2033	S	S	E	N	T	Y	K	S	R	R	R	S	Y	H	S	S		2048
6145	TTG	CGG	CTG	TCA	GCC	CAC	CGC	CTG	AAC	TCT	GAT	TCA	GGC	CAC	AAG	TCT		6192
2049	L	R	L	S	A	H	R	L	N	S	D	S	G	H	K	S		2064
6193	GAC	ACT	CAC	CGC	TCA	GGG	GGC	AGG	GAG	CGG	GGG	CGA	TCA	AAA	GAG	CGA		6240
2065	D	T	H	R	S	G	G	R	E	R	G	R	S	K	E	R		2080
6241	AAG	CAT	CTT	CTC	TCT	CCT	GAT	GTC	TCC	CGC	TGC	AAT	TCA	GAA	GAG	CGA		6288
2081	K	H	L	L	S	P	D	V	S	R	C	N	S	E	E	R		2096
6289	GGG	ACC	CAG	GCT	GAC	TGG	GAG	TCC	CCA	GAG	CGC	CGT	CAA	TCC	AGG	TCA		6336
2097	G	T	Q	A	D	W	E	S	P	E	R	R	Q	S	R	S		2112
6337	CCC	AGT	GAG	GGC	AGG	TCA	CAG	ACG	CCC	AAC	AGA	CAG	GGC	ACA	GGT	TCC		6384
2113	P	S	E	G	R	S	Q	T	P	N	R	Q	G	T	G	S		2128
6385	CTA	AGT	GAG	AGC	TCC	ATC	CCC	TCT	GTC	TCT	GAC	ANC	AGC	ACC	CCA	AGA		6432
2129	L	S	E	S	I	P	S	V	S	D	X	S	T	P	R			2144
6433	AGA	AGT	CGT	CAG	CTC	CCA	CCC	GTC	CCG	CCA	AAG	CCC	CGG	CCC	CTC			6480
2145	R	S	R	Q	L	P	P	V	P	P	K	P	R	P	L			2160
6481	CTT	TCC	TAC	AGC	TCC	CTG	ATT	CGA	CAC	GGC	GGC	AGC	ATC	TCT	CCA	CCT		6528
2161	L	S	Y	S	S	L	I	R	H	A	G	S	I	S	P	P		2176
6529	GCT	GAT	GGA	AGC	GAG	GGC	TCC	CGG	CTG	ACC	TCC	CAA	GCT	CTG	GAG			6576
2177	A	D	G	S	E	E	G	S	P	L	T	S	Q	A	L	E		2192
6577	AGC	AAC	AAT	GCT	TGC	CTG	ACC	GAG	TCT	TCC	AAC	TCT	CCG	CAC	CCC	CAG		6624
2193	S	N	N	A	C	L	T	E	S	S	N	S	P	H	P	Q		2208
6625	CAG	AGC	CAA	CAT	GCC	TCC	CCA	CAG	CGC	TAC	ATC	TCC	GAG	CCC	TAC	TTG		6672
2209	Q	S	Q	H	A	S	P	Q	R	Y	I	S	E	P	Y	L		2224
6673	GCC	CTG	CAC	GAA	GAC	TCC	CAC	GCC	TCA	GAC	TGT	GGT	GAG	GAG	ACG			6720
2225	A	L	H	E	D	S	H	A	S	D	C	G	E	E	E	T		2240

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6721	CTC	ACT	TTC	GAA	GCA	GCC	GTG	GCT	ACT	AGC	CTG	GGC	CGT	TCC	AAC	ACC	6768
2241	L	T	F	E	A	A	V	A	T	S	L	G	R	S	N	T	2256
6769	ATC	GGC	TCA	GCC	CCA	CCC	CTG	CGG	CAT	AGC	TGG	CAG	ATG	CCC	AAC	GGG	6816
2257	I	G	S	A	P	P	L	R	H	S	W	Q	M	P	N	G	2272
6817	CAC	TAT	CGG	CGG	CGG	AGG	CGC	GGG	CCT	GGG	CCA	GGC	ATG	ATG	TGT	6864	
2273	H	Y	R	R	R	R	R	G	G	P	G	P	G	M	M	C	2288
6865	GGG	GCT	GTC	AAC	AAC	CTG	CTA	AGT	GAC	ACG	GAA	GAT	GAC	AAA	TGC	6912	
2289	G	A	V	N	N	L	L	S	D	T	E	E	D	D	K	C	2304
6913	TAG	AGG	CAG	C													6922
2305	*	R	Q														2307

Fig. 1 (11 cont'd)

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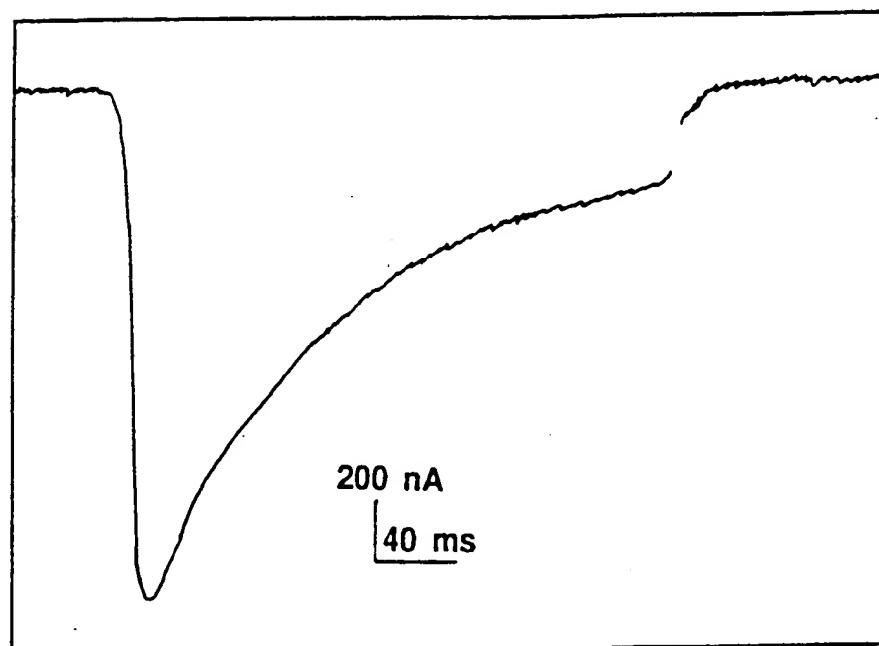


Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/08589

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/12, 15/63, 5/10; C12Q 1/02
US CL :536/23.5; 435/320.1, 240.2, 4; 530/350, 395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5; 435/320.1, 240.2, 4; 530/350, 395

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 260, issued 21 May 1993, T. W. Soong et al., "Structure and Functional Expression of a Member of the Low Voltage-Activated Calcium Channel Family", pages 1133-1136, especially the abstract, introduction, Fig. 1, and footnotes nos. 9 and 11.	1-10
Y	Genbank sequence database record, Accession no. L15453, issued 28 May 1993. see the entire record.	1-10

Further documents are listed in the continuation of Box C.

See patent family annex.

•	Special categories of cited documents:	
•A•	document defining the general state of the art which is not considered to be of particular relevance	•T• later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
•E•	earlier documents published on or after the international filing date	•X• document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
•L•	document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	•Y• document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
•O•	document referring to an oral disclosure, use, exhibition or other means	
•P•	document published prior to the international filing date but later than the priority date claimed	•S• Document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

03 OCTOBER 1994

Authorized Officer

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/08589

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Neuron, Volume 8, issued January 1992, M. E. Williams et al., "Structure and Functional Expression of α -1, α -2, and β Subunits of a Novel Human Neuronal Calcium Channel Subtype", pages 71-84, especially the abstract, Fig. 1, and the paragraph bridging pages 71-74.	1-10
Y	Science, Volume 231, issued 07 March 1986, N. Dascal et al., "Expression and Modulation of Voltage-Gated Calcium Channels After RNA Injection in Xenopus Oocytes", pages 1147-1150, especially the abstract, Figures 1 and 2, and the introduction.	4, 5, 7-10
A	EP, A2, 0,507,170 (FRANZ et al.) 07 October 1992; see especially Sequence No. 27980/2.	1-10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/08589

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

USPTO-APS, Medline, Biosis, CAS, Derwent WPI

Search terms: calcium channel; alpha-1c; human; clon?, recombinant?, cDNA

IgSuite Sequence databases